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In a search for genes with a role in hormonal control of cell proliferation, we cloned a novel progestin-induced gene, EDD (previously DD5) that appears to be the human homologue of the *hyd* tumor suppressor gene of *Drosophila melanogaster*. By amino acid homology with other known proteins it is likely that the EDD protein is an E3 ubiquitin-protein ligase, enzymes which target one or more key proteins for destruction by ubiquitin-mediated proteolysis. The interaction between EDD and two potential ubiquitination substrates was further confirmed. EDD was found to contain 2 nuclear localization signals both of which confer ability to interact with the nuclear import molecule importin α1. This was supported by the expression of green fluorescent protein-EDD fusion protein visualised predominantly in the nucleus of several cell types. A new system for inducible mammalian expression of EDD was tested and EDD cDNA from 26 breast, ovarian and prostate cancer cell lines was sequenced to determine the frequency of mutations in the EDD gene. Two high quality publications will result from this work.

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ANNUAL SUMMARY- Year 3

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INTRODUCTION

In a search for genes with a role in hormonal control of cell proliferation, we have cloned a novel progestin-induced gene, EDD (previous designation DD5). It is hypothesized that EDD has a role in the control of cell growth and differentiation as it appears to be the human homologue of the *hyperplastic discs (hyd)* gene of *Drosophila melanogaster*. Mutations in *hyd* are either lethal or cause abnormal or hyperplastic growth in *Drosophila* larval imaginal discs and defective germ cell development. While the biochemical roles of *hyd* and EDD are unknown, by amino acid homology with other known proteins it is likely that the EDD protein is a ubiquitin-protein ligase (E3), enzymes which target one or more key proteins for destruction by ubiquitin-mediated proteolysis. The target proteins of EDD would be expected to have profound effects on cell cycle control or cell signalling. The project is designed to :

- define the normal function of EDD and its targets and relate this role to development and progression of breast cancer;
- determine the effects of EDD on breast cancer cell cycle progression;
- determine the role of EDD in progestin-induced growth stimulation of breast cancer cells; and
- determine the effect of mutations in EDD or dysregulation of EDD expression on tumor phenotype and tumor progression.

Over the course of the project significant progress was made towards these aims with demonstration of ubiquitin ligase properties for EDD and the identification of several potential target proteins in the first two years. In year 3 these interactions were confirmed and studied in more detail. The significance of the interaction between EDD and importin α1 appears to lie in the correct localization of EDD within the cell and indeed GFP-EDD fusion proteins localized largely to the nuclei of several cell types. This observation has enabled us to tailor future experiments appropriately by focusing on a nuclear role for EDD and this work will be published in a high ranking international journal.

Over the grant period a number of different systems have been tested for EDD over or under expression, namely different constitutive promoters in different cell lines, the Tet and ΔMT inducible systems and antisense approaches. Many of these approaches were plagued with problems associated with the over-expression of a very large protein of integral cellular function. Perhaps the most useful mammalian system will prove to be the EDD knockout mouse model, in which we already know that EDD is essential for embryonic development and possibly for cell survival. Any breast cancer cell model for EDD over or under expression that we develop in the future will be an important complement to the mouse model.

A new role for EDD emerged in year 1 and this formed the basis of a successful grant proposal and promises to shed light on the role of EDD in the progesterone response. On this note, a progesterone receptor GST fusion protein was shown to interact with EDD protein *in vitro*. Another exciting development was demonstration of disruption around the EDD genetic locus in several cancers. This work has been extended to breast cancers as part of an ongoing project and the outcomes form the basis of a manuscript for publication in the specialist journal Cancer Research.

BODY OF REPORT

TASK 1: To determine the ability of EDD protein to form a thioester bond with ubiquitin *in vitro*.

This work was completed and published in year 1.

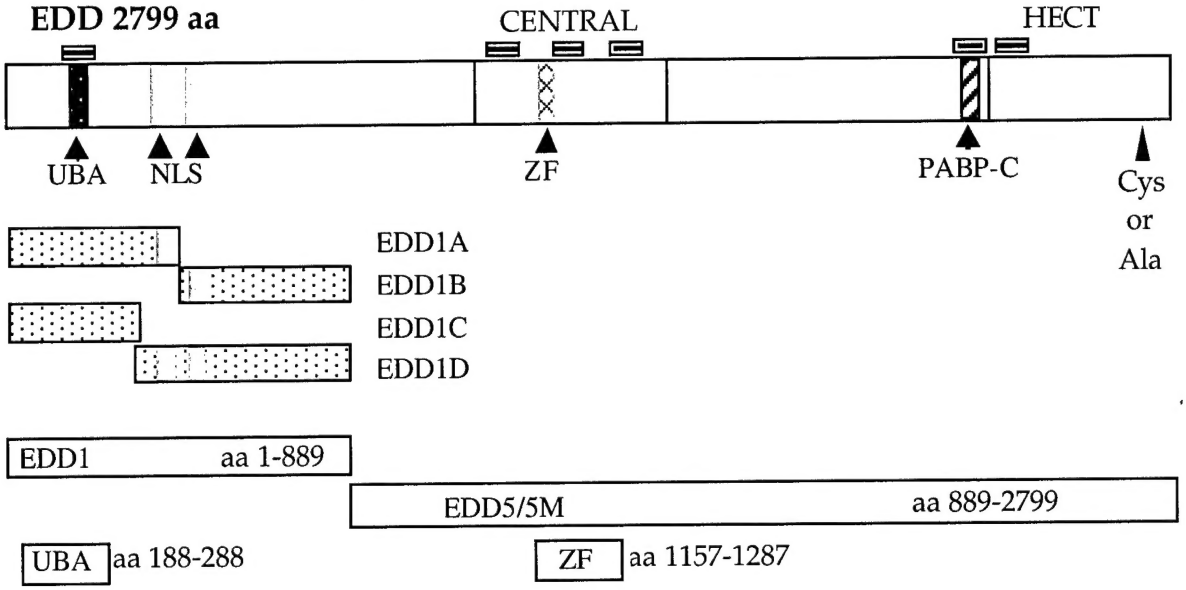

Figure 1. Schematic representation of the EDD protein sequence and overlapping deletion mutants.

Shown are the two domains highly conserved between EDD and HYD (CENTRAL and HECT) and the conserved cysteine residue (Cys) which has been mutated to alanine (Ala) in the ubiquitination-defective mutant. The positions of five receptor-binding LXXLL motifs are indicated as horizontal black bars, along with two putative nuclear localization signals (NLS). The products of four new constructs for *in vitro* translation to map interaction with importin α1 are designated EDD1A-1D. Newly generated yeast two-hybrid bait constructs are indicated in the lower half of the diagram: EDD1, N-terminal third; EDD5 or EDD5M, C-terminal two-thirds either wildtype (EDD5) or Cys to Ala mutant (EDD5M). Other domains indicated are: UBA, ubiquitin associated domain; ZF, RING-like zinc finger domain and PABP-C, region of homology to polyA binding protein C-terminus. aa, amino acids.

TASK 2: To identify substrates for EDD-mediated ubiquitination.

The main strategy for isolation of EDD-interacting proteins was to use yeast two hybrid library screening. Two human cDNA libraries, both cloned into the pACT2 vector, were available for screening in our laboratory, one derived from human placental mRNA and another derived from breast carcinoma mRNA. In year one, full length EDD cDNA was cloned downstream of the GAL4 DNA-binding domain in the pAS2.1 vector and used to screen three million clones from the placental cDNA library for interacting proteins. The C2768A mutant form of EDD was used to prevent degradation of potential interacting substrates. Two potentially interacting proteins, the human homologue of yeast SRP-1 (importin α1) and calcium-integrin binding protein (CIB), also known as kinase interacting protein (KIP), were identified as proteins which enable reconstitution of GAL4 promoter-binding activity, thus driving *HIS3* and *LacZ* reporter gene expression in the yeast strain Y190. Importin α1 is a protein essential for nuclear transport and may regulate passage of EDD between the nucleus and cytoplasm, while CIB/KIP appears to be involved in cell signalling and also interacts with DNA-dependent protein kinase, suggesting a second, nuclear role.

By GST pull down it was shown previously that importin α1 interacted with the N-terminal region of EDD which contains two potential nuclear localization signals (NLS). Additional constructs were designed and generated for *in vitro* translation of EDD protein to determine which of these NLS were important for interaction (Fig 1). The results indicated that either NLS could convey binding between EDD and importin α1 but that binding was strongest when both NLS were present. To test whether EDD was indeed located in cell nuclei, a construct for mammalian expression of an EDD-GFP (green fluorescent protein) fusion protein was generated in our lab. When the fusion protein was expressed in MCF7 breast cancer cells or HEK-293 human kidney cells, green fluorescence indicated an exclusively nuclear localization for EDD. In two other cell lines, T-47D breast cancer cells and CHO (chinese hamster ovary) cells, EDD-GFP expression was predominantly nuclear. These results

will need to be corroborated with immunostaining of endogenous EDD protein. To date the available antisera for EDD have proven unsuitable for this technique. However we have raised additional antibodies which are useful for both western blotting and/or immunoprecipitation and these are currently under trial for use in immunostaining of cells.

The interaction between CIB/KIP had been previously confirmed by GST-CIB pull down of EDD *in vitro* translated protein. We now have evidence that this interaction can occur in cells. A construct was made for mammalian expression of a FLAG-tagged CIB/KIP fusion protein. When this protein was expressed in cells stably over-expressing EDD protein, CIB/KIP and EDD could be co-immunoprecipitated from the cell lysates using the FLAG antibody. Together with the characterisation of the interaction with importin α1, this work forms part of a manuscript in preparation for publication.

Yeast two-hybrid screening to date had yielded a small number of EDD interacting proteins. More might be expected given the presence of several domains likely to function in protein-protein interaction including the HECT domain, the RING-like Zinc Finger (ZF) and a polyA-binding protein carboxy terminus homology region, as well as a ubiquitin associated domain (UBA), of unknown function. Various new Gal4DBD fusion baits were generated to cover these regions (Fig. 1). Specifically, these are EDD5 (aa 889-2799wt), EDD5M (aa 889-2799 C2768A mutant), UBA (aa 188-288) and ZF (aa 1157-1287). When the UBA domain was expressed as a fusion protein with the Gal4DBD this bait was found to autoactivate expression of the *LacZ* reporter gene and therefore could not be used in this system. The UBA motif may represent a transcriptional activation domain within EDD. The other three baits were exhaustively screened against a breast tumor cDNA library using two different yeast host strains. The ZF bait was also screened against the previously used placenta cDNA library. The numbers of clones screened and numbers positive for *LacZ* gene expression are listed in Table 1. The repeated isolation of CIB/KIP was testament to a functioning screening technique. However all other clones isolated turned out to be false positives. The ZF bait was found to associate with many library proteins in a non-specific manner and thus gave a very high background in the screens. It was thus concluded that an alternative method for isolating proteins that associate with EDD should be pursued. Future work will utilise EDD antisera for isolation of nuclear protein complexes followed by sequencing of the protein components of these complexes by mass spectrometry, a technique which has been optimised in our laboratory.

cDNA SOURCE	BAIT	YEAST STRAIN	NO. OF CLONES SCREENED	POSITIVES
YEAR1 and 2				
Placenta	EDDM	Y190	3.3×10^6	4 (2+2)*
Placenta	EDD3M	Y190	5×10^5	0
Placenta	EDD3M	Y190	2×10^6	0
Placenta	EDD2	Y190	2×10^6	6
YEAR3				
Breast Tumor	EDD5M	Hf7c	1.3×10^5	1
Breast Tumor	EDD5M	Hf7c	2×10^6	0
Breast Tumor	EDD5	Hf7c	1.3×10^5	0
Breast Tumor	EDD5M	Hf7c	2.3×10^6	0
Breast Tumor	EDD5	Y190	4×10^6	15
Breast Tumor	EDD5M	Y190	1×10^6	12^
Breast Tumor	ZF	Y190	6×10^5	>100
Placenta	ZF	Y190	8×10^5	>100

Table 1. Summary of yeast two-hybrid screening.
Baits are as described in Fig.1. * 2 positives isolated encoded CIB/KIP and 2 encoded importin α1. ^ Including one clone for CIB/KIP.

TASK 3: To determine the effects of EDD under- and over-expression on cultured breast cancer cells.

If EDD is a tumor suppressor gene we predict that over-expression of EDD in breast cancer cells should affect their growth. Using FLAG-tagged EDD expression vectors in T-47D cells we monitored the effect of EDD on the growth rate of cells by measuring their ability to form colonies. We used transient transfection of EDD or vector alone and selected for transfectants using the puromycin resistance gene system. However we found that the presence of EDD in the test population caused up-regulation of the puromycin resistance gene (most likely due to the general co-activation properties of EDD that we have observed), leading to a greater number of colonies surviving. This phenomenon would have masked any growth effects and we were unable to pursue these experiments further.

Considerable difficulty has been encountered when developing an inducible expression system for EDD. The development of such a system would enable us to ask many questions about the effect of EDD on cell survival. Previously we have been able to use a human embryonic kidney cell line, HEK-293, transfected with EDD constructs in the pRcCMV constitutive vector. As reported last year we established several stable cell lines over-expressing either EDD or ubiquitination defective EDD, as well as empty vector transfected lines as controls. These lines have already proven a valuable resource for characterising the EDD protein and for co-immunoprecipitation studies. For example they were used to confirm interaction of EDD with importin α1 and CIB/KIP. During the past year further attempts to develop and inducible expression system for EDD in mammalian cells. This involved cloning a cDNA encoding FLAG-tagged EDD into the vector ΔMT, a vector for mammalian expression drive by a zinc-responsive promoter. Inducible expression was tested in several cell lines, including HEK-293, the breast cancer cell lines T-47D and MCF7, and two ovarian cancer cell lines, but no expression was detectable. During the next two months we will be testing a system where EDD cDNA expression is driven by a promoter system responsive to the ecdysone analogue, ponasterone (Clontech). In the absence of an inducible system we propose to use RNA interference to shut down EDD expression in various cell types and note the effects on cell cycle and cell survival. Along this line, we now have available a knockout mouse model, the phenotype of which is embryonic lethal. Cells isolated from knockout embryos will be used to study the defects associated with an absence of EDD protein.

We have used various agents to halt or synchronize cells at different cell cycle stages but so far have not seen any significant changes in EDD protein levels. Next we will look at whether the cellular location or phosphorylation state of EDD changes in response to any of these agents.

TASK 4: To determine the expression of EDD in breast tumor specimens.

Initial attempts to perform immunohistochemistry on paraffin embedded cell blocks were not successful due to a high level of background staining by the antisera. The antisera was affinity purified and tested through collaborations with a local laboratory. The affinity purified antibody was also found to be unsuitable for immunohistochemistry. We have now raised additional antisera in sheep and these are currently being tested for their suitability for immunohistochemistry.

Previously we used microsatellite allelotyping of DNA extracted from tumors and matching normal tissues to determine the presence of chromosomal aberrations such as loss of heterozygosity (LOH) at the EDD locus on 8q22.3. We demonstrated that allelic imbalance occurred at high frequency, particularly involving a microsatellite within the EDD gene, in ovarian cancers (notably in the serous subtype), hepatocellular carcinomas, squamous cell carcinomas of the tongue and metastatic melanoma. These results are exciting as they are consistent with the presence of a tumor suppressor gene at or very near the EDD locus and suggest that the EDD gene may have a common role in the progression of several human cancers.

Where allelic imbalance is due to LOH, it is expected that the remaining copy of the EDD gene might contain an inactivating mutation. The next stage of this project therefore involved developing a sequencing strategy for EDD cDNA from cell lines and tumors. To make an assessment of the frequency of mutations in the EDD gene in cancer, the complete coding region of the EDD mRNA (8397 nucleotides) was sequenced using cDNA derived from 26 breast, ovarian and prostate cancer cell lines. Three normal breast epithelial cell lines were also sequenced. A list of the sequence variants found is shown in Table 2. Only 3/26 cancer cell lines had mutations in EDD mRNA that resulted in a change to the translated amino acid sequence. These missense mutations were confirmed in the genomic sequence. The amino acid changes were not in regions of the EDD protein with any obvious functional motifs and only the His>Asn mutation in the SK-Br-3 line alters amino acid polarity. Few polymorphisms or silent substitutions were observed. Of the six conservative sequence variants, at least five are likely to be polymorphisms as they are either found in RNA derived from normal cell

lines or tissues, or in multiple cell lines. A splice variant was also observed in all cell lines. The variant differs from the full length EDD mRNA by deletion of 18bp of sequence from nt 884-901, removing the amino acid sequence VLLLPL. Although this motif is not located in any of the putative functional domains of EDD, removal of this sequence does have the potential to disrupt protein structure and might alter enzymatic function or localization of the protein.

SSCP analysis of 29 ovarian tumors, 37 breast tumors and 29 colon tumors confirmed the low frequency of mutation of the EDD gene, although only eight exons of EDD (covering approximately 13% of the coding sequence) were studied. These exons encode one N-terminal nuclear localization signal (nt 1482-1598), the zinc finger motif (nt 3573-3812) and the majority of the HECT domain (nt 7602-8339). No mutations were found in the coding regions or splice junction sites of any tumor. The above work forms part of a manuscript soon to be submitted to the journal Cancer Research. The paucity of polymorphisms and mutations in a gene of this size from a range of cancer cell lines is quite remarkable and perhaps underscores the low tolerance of cells for null mutations in EDD. This has been borne out by the lethal phenotype of mice lacking a functional EDD protein and appears to be a property of an emerging class of tumor suppressor genes (see Liu *et al*, 2000, Genes Dev 14:1448, for example).

Nucleotide postion[1]	Codon	Base change	Predicted amino acid change	Cell line(s)
4753	**1584**	**C→A**	**His→Asn**	**SK-BR-3**
6279	**2093**	**A→G**	**Asn→Ser**	**IGROV-1**
2468	**823**	**C→T**	**Ala→Val**	**OVCAR-8**
886-902	296-300	Splice variant	VLLPL removed	Detected in all cell lines
7689		A→C	No change	Hs 578T
4055		C→T	No change	BT-20 Human mRNA
4390		A→G	No change	Human mRNA
4556		A→G	No change	T-47D MDA-MB-134
3956		A→G	No change	HMEC-184 T-47D MCF-7 BT-549 ZR-75-1 MDA-MB-134 OVCAR-8 DU-145 LnCap Hs 578T
7634		C→A	No change	HMEC-184 T-47D MCF-7 BT-549 DU-145 Hs 578T

Table 2. Summary of EDD sequence variants detected in 29 normal epithelial and cancer cell lines.

Base changes in bold confirmed at the genomic level. [1]Nucleotide numbering starts at the A of the initiation codon

APPENDICES

1. Key Research Accomplishments

- Generation of Flag tagged plasmid constructs for EDD and yeast two-hybrid positives suitable for for over-expression in mammalian cell lines.
- Evidence for *in vivo* interaction between EDD and CIB/KIP in mammalian cells.
- The N-terminal region of EDD was found to contain 2 NLS, both of which interact with importin α1.
- Generation of GFP-tagged EDD constructs and use of these to ascertain the cellular location of the EDD protein in several cell lines. New extraction techniques were developed to enrich lysates for nuclear proteins.
- Several assays were tested as systems to ascertain whether CIB/KIP might be a ubiquitination target of EDD. Unfortunately the answer to this question still awaits identification of an E2 enzyme partner for EDD.
- Screening of breast cancer and placenta cDNA libraries for interacting proteins using 4 new EDD baits. As confirmation, a previously isolated positive, CIB/KIP, was again detected in such screen.
- Production of new antisera reactive against EDD protein and demonstration of their utility for use in immunoprecipitation and western blotting protocols.
- Construction of ΔMT vector constructs for inducible expression of EDD in mammalian cells.
- EDD cDNA from 26 breast, ovarian and prostate cancer cell lines was sequenced and remarkably low numbers of mutations and polymorphisms noted.

2. Reportable Outcomes

Poster presentations

1. Characterisation of EDD: a progestin-regulated tumor suppressor gene.
Michelle J. Henderson, Jennifer L. Clancy, Amanda J. Russell, Gillian M. Lehrbach, Robert L. Sutherland and Colin K. W. Watts.
11th International Congress of Endocrinology, October 29-November 2, 2000, Sydney, NSW, Australia.

2. Functional characterisation of EDD, a progestin-regulated HECT E3 ligase.
Michelle J. Henderson, Amanda J. Russell, Gillian M. Lehrbach, Samantha L. Hird, Marcia Munoz, Robert L. Sutherland and Colin K. W. Watts.
Hormones and Cancer 2000, November 3-7, 2000, Port Douglas, QLD, Australia.

3. Characterisation of EDD: a progestin-regulated gene with dual functions.
Michelle J. Henderson, Jennifer L. Clancy, Amanda J. Russell, Gillian M. Lehrbach, Samantha L. Hird, Marcia Munoz, Robert L. Sutherland and Colin K. W. Watts.
22nd Annual Conference on the Organisation and Expression of the Genome, February 11th-15th, 2001, Lorne, VIC, Australia.

Papers in preparation

1. Amplification and overexpression of EDD, the human *hyperplastic discs* gene, is frequent in cancer. J. Clancy[1], M.J. Henderson[1], A.J. Russell[1], D.W. Anderson[2], R.J. Bova[1], I.G. Campbell[3], G.A. MacDonald[4], G.J. Mann[5], T. Nolan[6], O.I. Olopade[7], R.L. Sutherland[1] and C.K.W. Watts[1]. For submission to Cancer Research in August 2001

2. EDD, the human hyperplastic discs protein, interacts with importin _1 and regulates progesterone receptor transactivation. Michelle J. Henderson[1], Amanda J. Russell[1], Samantha Hird[1], Marcia Munoz[1], Gillian M. Lehrbach[1], David A Jans[2], Robert L. Sutherland[1] and Colin K.W. Watts[1,3] For submission to Journal of Biological Chemistry in September 2001

Research Opportunities

The progress made in this project resulted in the participation of the PI in national and international scientific conferences. In addition, important collaborative ties in several areas have been made possible. For example, attendance at the Era of Hope Meeting in June 2000 led to establishment of a collaboration that in turn has shed light on one of the possible functions for EDD.

This award has also made possible substantial career development for the postdoctoral trainee. This has been in the form of added responsibility for the project, supervision of staff and students, successful application for grants and preparation of publications and reports, in addition to development of new technical expertise and research management skills.